

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<b>Applicants:</b> Robin Robinson <i>et al.</i>	)	
	)	
<b>Serial No.:</b> 10/617,569	)	<b>Confirmation No.:</b> 5752
	)	
<b>Filed:</b> July 11, 2003	)	<b>Group Art Unit:</b> 1648
	)	
<b>For:</b> Functional Influenza Virus-Like Particles (VLPS)	)	<b>Examiner:</b> Myron G. Hill
	)	

DECLARATION OF GALE SMITH, PH.D. UNDER 37 C.F.R. § 1.132

I, Dr. Gale Smith, hereby declare as follows:

1. I am currently employed as Vice-President of Vaccine Development at Novavax, Inc. I received my Ph.D. in 1983 from Texas A&M University. Prior to Joining Novavax, I was Chief Scientific Officer for Protein Sciences Corporation and was a Research Scientist at Texas A&M University. My resume is attached.
2. I have reviewed the office action and understand the scientific and legal conclusions drawn by the examiner. I have reviewed the Latham *et al.* reference (*Journal of Virology*, Vol 75, pages 6154-6165) and understand its teachings.
3. The instant application discloses virus-like particles (VLPs) comprising avian influenza virus M1 proteins from A/Hong Kong/1073/99 (H9N2). In contrast, the VLPs disclosed by Latham *et al.* comprise M1 proteins from the seasonal human influenza virus strain A/Udorn/72 (H3N2).
4. In experiments performed under my supervision, I have observed the unexpected result that the production of VLPs comprising M1 derived from avian influenza virus strains is far superior to the production of VLPs comprising M1 derived from human influenza strains.

a. For example, in one set of experiments described in my declaration filed December 3, 2007, seasonal and avian M1 and HA proteins were cloned and expressed in a baculovirus expression system. The seasonal influenza strains used for these experiments were A/Fujian/411/02 and A/Wisconsin/67/05 while the avian strain used was A/Indonesia/5/05.

b. Cells expressing either seasonal M1 and HA, avian M1 and HA or a combination of seasonal and avian M1 and HA were grown under conditions that allow formation of VLPs.

c. The VLPs were harvested and isolated from the supernatant by centrifugation and by a discontinuous sucrose step gradient. The fraction comprising the VLPs was collected from the middle of the gradient at about 40% sucrose.

d. Exhibit 1 is an SDS-PAGE and western immunoblot showing the VLPs isolated from the sucrose gradient. The lanes in the gel comprise the following: 1 to 5, A/Fujian M1 (seasonal) with 4 different HAs or alone; 6 to 10, A/Indonesia M1 (avian) with 4 different HAs or alone; 11 to 14, various controls.

e. Comparing the bands on the gel, the lanes that comprise VLPs comprising avian M1 have stronger bands of M1 and HA in the same lanes, while the lanes that comprise seasonal influenza have weaker bands in the gel. M1 and HA bands in the same lane is indicative of HA associating with M1. This association is indicative of VLP formation comprising M1 and HA. These data provide evidence that VLPs comprising the avian influenza M1 form more efficiently than seasonal influenza M1, with either homologous or heterologous envelope proteins.

5. To identify the structural elements within avian M1 proteins responsible for the increase in VLP production as compared to seasonal M1 proteins, the M1 amino acid sequences of three avian influenza strains were aligned with the M1 sequences from a variety of seasonal and

pandemic human influenza strains (Exhibit 2). The alignments revealed that avian influenza virus strains contain the sequence "YKKL" at amino acids 100-103 of the M1 protein. In contrast, human influenza M1 proteins harbor "YRKL" at amino acids 100-103 of the M1 protein. These four amino acids represent a motif called the late domain (L-domain) which is important in recruiting host components required for budding and release of virus particles.

a. As shown in Exhibit 3, the avian influenza strain disclosed in the present application, A/Hong Kong/1073/99, also possesses the "YKKL" L-domain sequence (M1 gene sequence shown in SEQ ID NO:3). On the other hand, the M1 protein from the human influenza virus strain A/Udorn/72 as described by Latham *et al.* contains the "YRKL" L-domain sequence.

6. To evaluate the significance of the YKKL L-domain, site-directed mutagenesis experiments were performed using the human seasonal strain A/Fujian/411/02 containing the YRKL L-domain sequence. Exhibit 4 shows the amino acid changes in seven A/Fujian mutants generated by site-directed mutagenesis.

a. Mutants 3 and 7 with an R101K mutation (and thus harboring the avian-like M1 YKKL L-domain sequence) were able to secrete significantly larger amounts of M1 from infected cells compared to strains possessing the seasonal-like M1 YRKL L-domain sequence (Exhibit 5). Comparing the bands on the gel, mutant 3 (lanes 3 and 10) and mutant 7 (lanes 7 and 14) show stronger bands than the other mutants, indicating an increased amount of M1 available for association in a VLP. These data demonstrate that mutants harboring the R101K mutation (and thus possessing the avian-like YKKL L-domain) show higher levels of M1 present in the pellet (secreted) fraction than mutants containing the YRKL (seasonal-like) domain.

7. The role of the avian YKKL L-domain sequence in increased VLP formation was confirmed using co-infection experiments (Exhibit 6).

- a. Sf9 cells were co-infected with baculovirus expressing A/Fujian hemagglutinin (HA) and neuraminidase (NA), in conjunction with recombinant baculovirus expressing either the avian influenza strain A/Indonesia M1, the wild-type human influenza strain A/Fujian M1, or the “repaired” Fujian M1 mutant, A/Fujian mutant (R101K) which was mutated to mimic the avian YKKL L-domain sequence. As a control, Sf9 cells were infected individually with each recombinant construct in the absence of A/Fujian HA and NA.
  - b. Higher levels of M1 were found in the pellet fraction from strains harboring the YKKL L-domain M1 sequence (see the stronger bands of YKKL-containing A/Indonesia in lanes 8, 11, 14 and YKKL-containing A/Fujian (R101K) in lanes 10 and 13) as compared to the YRKL-containing wild-type A/Fujian strain (see lanes 9 and 12). These results were confirmed with a western immunoblot (see Figure B in Exhibit 6). The increased intensity of the bands indicate that there is more M1, and thus, more VLPs.
  - c. Furthermore, strains harboring the YKKL L-domain M1 sequence (A/Indonesia and the repaired A/Fujian mutant (R101K)) showed higher levels of hemagglutination activity than the YRKL-containing wild-type (WT) A/Fujian/411/02 strain using turkey and guinea pig RBCs (see table in Exhibit 6 which shows the HA titer of each VLP sample using a hemagglutination assay).
8. As shown in Exhibit 7, VLP formation using M1 from the avian A/Hong Kong/1073/99 (H9N2) as specified in the instant application is far superior to that of M1 from the strain disclosed by Latham *et al.* (A/Udon/72 (H3N2)).
- a. Exhibit 7 shows an SDS-PAGE comparing M1s (including the Latham *et al.* A/Udon/72 M1) for the intrinsic ability (without co-expression with envelope proteins) to form VLPs. Proteins are stained with coomassie blue in sucrose gradient purified particles from Sf9 cells expressing: Lane 1: Human H3 A/Fujian M1 with the R101K mutation and more optimal YKKL late domain at position 100-103; Lane 2: Avian

A/Indonesia/5/05 M1; Lane 3: Human A/Udorn/72 (Latham *et al.*) M1; Lane 4: avian A/Hong Kong M1.

b. The yield of secreted M1 particles was determined by scanning the SDS-PAGE gel and normalizing the yield of M1 VLPs relative to avian A/Indonesia/5/05 M1 VLPS (lane 2). The YRKL L-domain containing Udorn M1 was very inefficiently secreted as particles (lane 3) at a level just 12% of the YKKL L-domain containing avian A/Indonesia/5/05 M1. This was not due to low expression of A/Udorn M1 as intracellular levels of these M1s were equivalent.

9. To determine the prevalence of the YKKL L-domain among influenza strains, BLAST searches were performed. The YKKL domain is found almost exclusively in influenza isolates from avian species. Out of 40 sequences analyzed, 39 are from avian species. The other is a tiger species.

10. These results demonstrate that the increased production of influenza VLPs seen with avian M1 is dependent on the sequence of the L-domain in the M1 protein. More specifically, the important difference between the seasonal human M1 and avian M1 is the L-domain sequence "YKKL", which found almost exclusively in avian influenza strains, including the A/Hong Kong/1073/99 (H9N2) strain disclosed in the instant application.

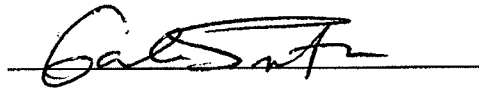
11. The structural feature found in avian M1 (the "YKKL" L-domain) results in an unexpected property that increases the level of VLP formation from Sf9 or other suitable cells. Accordingly, the M1 of avian influenza strains represents a better protein for the production of VLPs than the M1 of human influenza strains, such as the one disclosed by Latham *et al* which contain the YRKL L-domain sequence.

12. The increased formation and recovery of VLPs with avian M1 is critical to vaccine development. Using a human seasonal M1 protein such as the one disclosed by Latham *et al.* does not produce sufficient quantities of VLPs for use in a vaccine. The only way to make

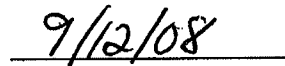
recoverable amounts of VLPs necessary for vaccine production is through the use of avian derived M1 proteins, such as those disclosed in the present application.

13. I declare that all statements made herein on my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Respectfully submitted,

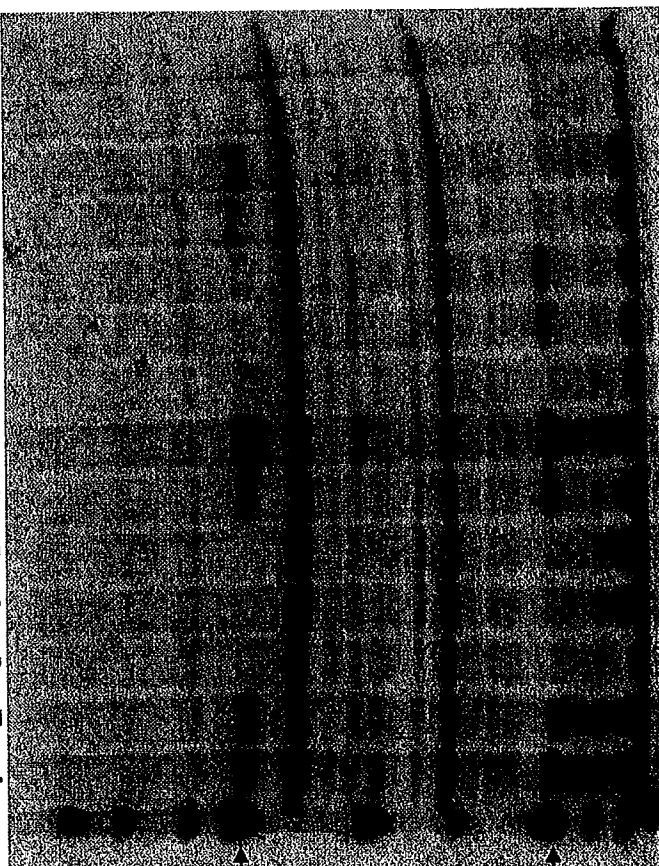


Gale Smith, Ph.D.



Date

EXHIBIT 1

Fujian M1					Indo M1									
1	2	3	4	5	6	7	8	9	10	11	12	13	14	
														
HA														
M1														

1. 245/230 VLP H3 Fujian HANAM1 +Fuj M1
2. 384/230 VLP H3 Wisc HA + Fuj M1
3. 281/230 VLP H1 NC HA + Fuj M1
4. 301/230 VLP H5 Indo HA + Fuj M1
5. 230 VLP Fuj M1
6. 245/299 VLP H3 Fujian HANAM1 + Indo M1
7. 384/299 VLP H3 Wisc HA + Indo M1
8. 281/299 VLP H1 NC HA + Indo M1
9. 301/299 VLP H5 Indo HA + Indo M1
10. 299 VLP Indo M1
11. 245 VLP H3 Fujian HANAM1
12. 384 H3 Wisc HA
13. 281 H1 NC HA
14. 301 H5 Indo HA

# EXHIBIT 2

A/Indonesia/505 M1	ms l i t e v e t y v i s i l p s p p k a e l a q k l e d r f a g k n d l e a l m e w l k t r p l i s p l t k g l - g f v t i t v p s e r g l q t r n r f v	78
A/Viet Nam/120304 M1		80
A/black-headed_goose/Cinghal/12005 M1		67
A/NY/5504 M1		80
A/Fujian/4112002 M1		80
A/Wisconsin/672005 M1		80
A/Wyoming/0303 M1		80
A/Arm Atbor/61960 M1		80
A/New Caledonia/2009 M1		80
A/Brevig Mission/11918 M1		80
A/PR/824 M1		80
A/New Caledonia/201999 M1		38
A/Indonesia/505 M1		190
A/Viet Nam/120304 M1		160
A/black-headed_goose/Cinghal/12005 M1		147
A/NY/5504 M1		160
A/Fujian/4112002 M1		160
A/Wisconsin/672005 M1		160
A/Wyoming/0303 M1		160
A/Arm Atbor/61960 M1		160
A/New Caledonia/2009 M1		160
A/Brevig Mission/11918 M1		160
A/PR/824 M1		160
A/New Caledonia/201999 M1		116
A/Indonesia/505 M1		239
A/Viet Nam/120304 M1		240
A/black-headed_goose/Cinghal/12005 M1		227
A/NY/5504 M1		240
A/Fujian/4112002 M1		240
A/Wisconsin/672005 M1		240
A/Wyoming/0303 M1		240
A/Arm Atbor/61960 M1		240
A/New Caledonia/2009 M1		240
A/Brevig Mission/11918 M1		240
A/PR/824 M1		240
A/New Caledonia/201999 M1		198



EXHIBIT 3

A_Indonesia_5_05 M1	mslltevetvylsiipsgplkaeiaqkledvfagkntdlealmewlktirpilspltkgi-gfvftltvpserglqrrrfv	20	40	60	80
A-Udom-307-72 M1	.....v.....r.....	100	120	140	160
A_Indonesia_5_05 M1	qnalngdpnmdravklvkkkkreiffhgakevslsystgalascmglllyrmgtvtteva fglvcatceqiadsqhr	180	200	220	240
A-Udom-307-72 M1	.....r.....ia.....a.....a.....				
A_Indonesia_5_05 M1	shrqmatitnplirhenrmvlasttakameqmagssesqaaeamevanqarqmvmamrtigtthpnssaglr dnl lenlqay				
A-Udom-307-72 M1	.....vat.....s.....a.....s.....k.d.....				
A_Indonesia_5_05 M1	qkrmgvqmqr f k	251			
A-Udom-307-72 M1	.....	252			

late domain

A/Indonesia/5/05	Tyr-Lys-Lys-Leu (YKKL)
A/Hong Kong/1073/99	Tyr-Lys-Lys-Leu (YKKL)
A/Udom/307/72	Tyr-Arg-Lys-Leu (YRKL)
Seasonal Flu	Tyr-Arg-Lys-Leu (YRKL)

EXHIBIT 4

Flu Fujian M1 Mutants

Opt Indo M1	K	N	N	A
	101	207	224	227
WT FJ M1	R	S	S	T
	101	207	224	227
FJ Mut 1 (S207N)	R	N	S	T
FJ Mut 2 (S224N)	R	S	N	T
FJ Mut 3 (T227A)	R	S	S	A
FJ Mut 4 (S224N, T227A)	R	S	N	A
FJ Mut 5 (R101K)	K	S	S	T
FJ Mut 6 (S207N, S224N, T227A)	S	N	N	A
FJ Mut 7 (R101K, S207N, S224N, T227A)	K	N	N	A

**EXHIBIT 5**  
**Expression of Flu Fujian M1 Mutants**

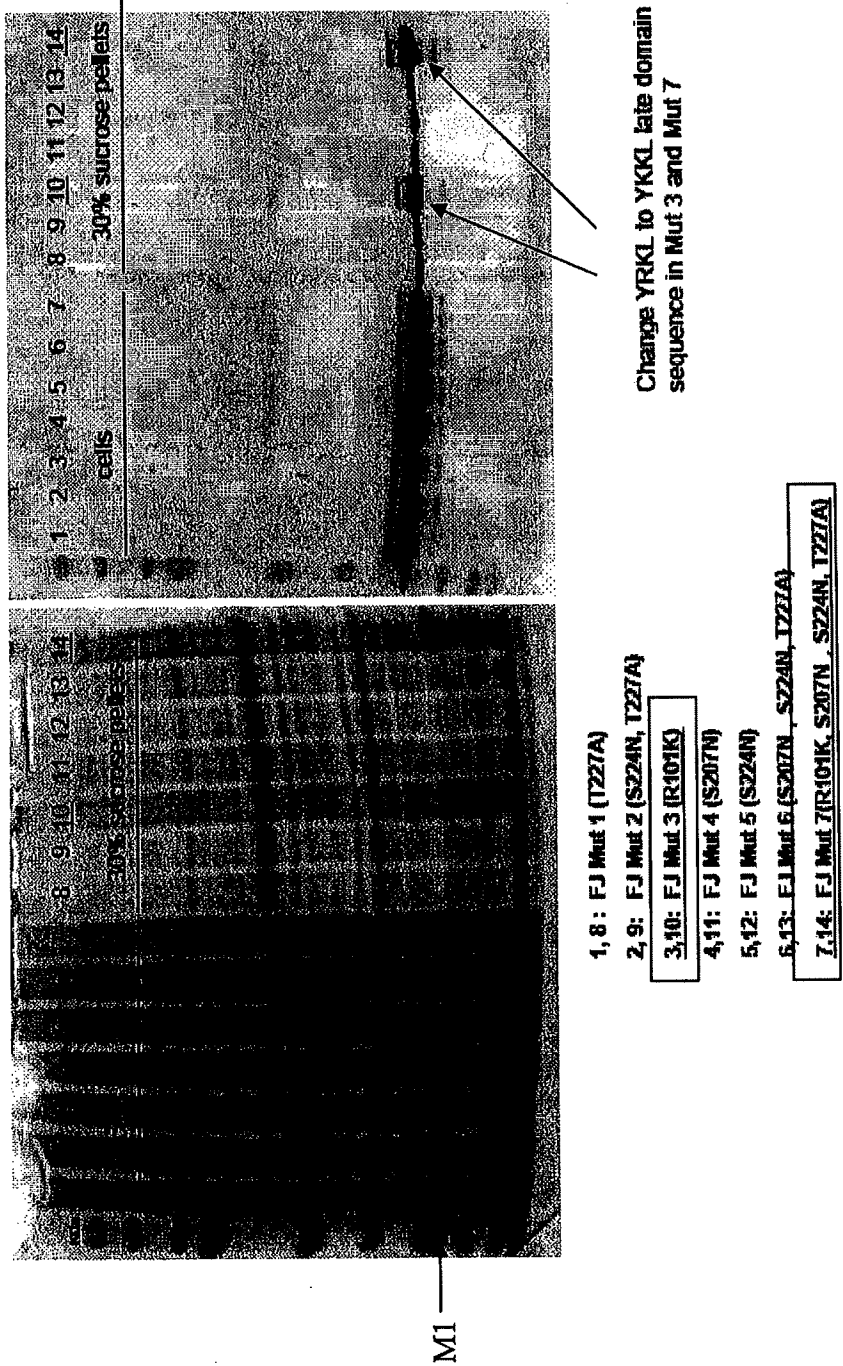
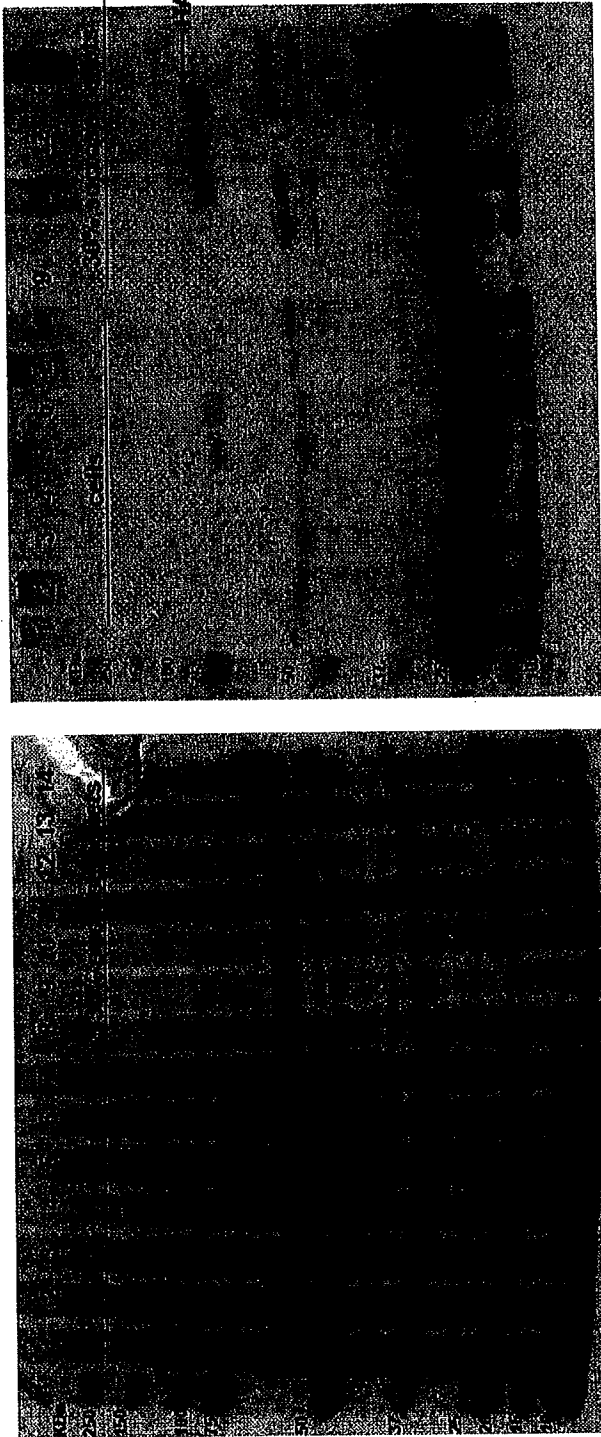


EXHIBIT 6

Co-infection of Flu M1 with Fujian HANA

(A)

(B)



Lane 1, Lane 8 : Indo M1  
Lane 2, Lane 9: WT Fujian M1  
Lane 3, Lane 10: Repaired Fujian M1 (R101K)  
Lane 4, Lane 11: Indo M1 co-infection with Fujian HANA  
Lane 5, Lane 12: WT Fujian M1 co-infection with Fujian HANA  
Lane 6, Lane 13: Repaired Fujian M1 (R101K) co-infection with Fujian HANA  
Lane 7, Lane 14: Indo HANAM1 as control

Co-infection VLPs	HA Turkey	HA G Pig
Indo M1/Fujian HANA (299.3.2 / 410.2.2)	2048	512
WT Fujian M1/Fujian HANA (230.1.2 / 410.2.2)	512	256
Repaired Fujian M1/Fujian HANA (561.3.1 / 410.2.2)	2048	512

EXHIBIT 7

Matrix VLP Production



Matrix Protein	Matrix VLPs*
Lane 1. H3 Fujian M1 R101K (YKKL late domain)	0.72
Lane 2. Indonesia M1	1.00
Lane 3. H3 Udorn M1	0.12
Lane 4. H9 HK M1	0.81

\*Relative yields M1 VLPs by scanning densitometry, normalized to Indonesia M1 (lane 2)